LC Troubleshooting

Flow Rate and Peak Spacing

Flow-rate changes might or might not be acceptable means to adjust retention.

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LC Troubleshooting Editor

Chromatographers can adjust six basic parameters to control liquid chromatography (LC) separations. They include mobile-phase composition, stationary-phase selection, temperature, packing particle size, column size, and flow rate. The last three parameters sometimes are called *column parameters* and are controlled by physical processes, whereas the first three are controlled by chemical processes.

In the past, workers took care to optimize the flow rate to get the maximum performance from a column because, for packing particles approximately 10 µm and larger in diameter, the column plate number (N) changes significantly with the flow rate. For example, a change in flow rate of 1–2 mL/min reduces N by approximately 18% for a 10-µm d_p column, whereas the change is roughly 10% for a 5-µm d_p column and roughly 4% for a 3-µm d_p column. Resolution varies with the square root of N, so a flow-rate change for 10-µm particles can make a noticeable change for poorly spaced peaks, but separations using smaller particles change only slightly. Today, chromatographers primarily use 3–5 µm d_p columns, and the flow rate is selected primarily for a convenient column back pressure.

Most LC methods have associated system-suitability tests. These tests often require that the retention time for an injected standard is within a certain retention time window. Some methods allow for adjustment in the flow rate to move peaks back within the desired window if system suitability is not met. This adjustment technique can be justified for isocratic separations because chromatographic selectivity, or relative peak spacing, does not change with flow rate when operating in the isocratic mode. Workers who habitually make such adjustments might make similar adjustments to gradient methods, not suspecting that these changes can have an adverse effect on the separation. The following discussion illustrates the difference between flow-rate adjustments with isocratic and gradient separations.

Isocratic Separation

The retention factor (k) can be used to describe retention in isocratic separation. Retention factor is defined as

$$k = \frac{t_R - t_0}{t_0} \qquad [1]$$

where t_R and t_0 are the retention and the column dead times, respectively. Changes in flow rate will change the retention and dead times proportionally. For example, a 10% reduction in flow rate will increase both values by 10%, so k remains unaffected by flow rate. This outcome is illustrated in Table I, in which data are presented for six peaks in an isocratic separation run at 2 mL/min and at 1 mL/min — k is unchanged when the flow rate is halved.

Selectivity (α) is the relative peak spacing in a separation. Equation 2 defines selectivity as

$$\alpha = \frac{k_2}{k_1} \qquad [2]$$

where k_1 and k_2 are the retention factors for the first and second peaks of a peak pair. Because k values are unaffected by flow rate, α will remain constant when flow rate is changed in isocratic separation. Table I shows this relationship in tabular form, and Figure 1 confirms it visually. A small — in this case almost unnoticeable — increase in resolution occurs when the flow rate is reduced. This change is caused by the influence of flow rate upon the column plate number, not the relative peak spacing. For situations in which the flow rate is changed to adjust retention time, changes of more than 10–20% in flow are rare, so it is unlikely that a change in resolution will be noticeable.

Table I: Comparison of performance parameters for an isocratic separation

Peak Number	t_R	k	α	Resolution
Isocratic 70% B, 2-mL/min flow rate				
1	2.90	1.3	1.27	4.78
2	3.34	1.6	1.10	2.09
3	3.55	1.8	1.05	1.15
4	3.67	1.9	1.28	5.77
5	4.33	2.4	1.13	3.20
6	4.74	2.7	—	—
Isocratic 70% B, 1-mL/min flow rate				
1	5.80	1.3	1.27	5.20
2	6.68	1.6	1.10	2.27
3	7.10	1.8	1.05	1.24
4	7.34	1.9	1.28	6.21
5	8.66	2.4	1.13	3.42
6	9.48	2.7	—	—

Table II: Comparison of performance parameters for a gradient separation

Peak Number	t_R	Average k	α	Resolution
20-min gradient, 2-mL/min flow rate				
1	13.47	3.1	0.76	0.97
2	13.64	4.1	1.32	1.48
3	13.9	3.1	0.91	1.08
4	14.08	3.4	1.00	1.37
5	14.32	3.4	1.06	0.94
6	14.47	3.2	—	—
20-min gradient, 1-mL/min flow rate				
1	18.85	1.5	0.94	2.33
3	19.31	1.6	0.76	0.75
2	19.46	2.1	1.24	0.71
4	19.62	1.7	1.00	1
5	19.82	1.7	1.06	0.52
6	19.93	1.6	—	—
40-min gradient, 1-mL/min flow rate				
1	26.95	3.1	0.76	1.17
2	27.29	4.1	1.32	1.77
3	27.81	3.1	0.91	1.3
4	28.17	3.4	1.00	1.64
5	28.63	3.4	1.06	1.13
6	28.95	3.2	—	—

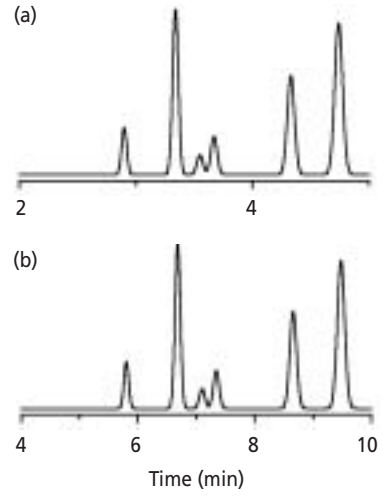

Figure 1: Isocratic separation of sample in Table I at (a) 2-mL/min and (b) 1-mL/min flow rates.

Gradient Elution

The situation is much different when flow rate is modified in gradient elution separations. Isocratic retention factors (k) change when the mobile-phase strength is changed. As long as isocratic conditions are maintained for a given mobile phase, k will remain constant. With gradient elution, the mobile-phase strength changes on a continuous basis, so k also will change during the separation. It is easy to see that k, as defined for isocratic separation, is an unsuitable measurement of retention for gradient elution. Instead, chromatographers should use an analogous term or average retention factor (k^*). Physically, this variable can be considered the instantaneous k value when a peak has traveled halfway through the column. Average retention factor is defined as

$$k^* = \frac{t_G F}{\Delta\%B\, V_m S} \qquad [3]$$

where t_G is the gradient time (for example, 20 min), F is the flow rate, Δ%B is the gradient range as a decimal (for example, 5–95% B would be 0.90), V_m is the column volume, and S is a characteristic of the analyte (for the present discussion, it can be assumed to be a constant value of 5 for all compounds).

From equation 3, it is easy to see the influence of flow rate on k^* in gradient elution. If the flow rate is halved from 2 mL/min to 1 mL/min, k^* is doubled. The data in Table II illustrate this relationship by comparing a 20-min gradient at 2 mL/min and 1 mL/min. For each compound, the k^* value doubles with the flow-rate change. Selectivity in gradient elution is defined in the same manner as in equation 2, except that k^* values for adjacent peaks are used instead of isocratic k values. Thus, it is not surprising to see that the relative peak spacing changes dramatically if only the flow rate is changed. By comparing Figures 2a and 2b and examining the data of Table II, you can see that not only does the peak spacing change, but peaks 2 and 3 reverse positions.

The reason that flow rate affects the separation so dramatically is that it has the same effect as changing the gradient steepness by changing the gradient time. To avoid changes in selectivity when changing the flow rate in gradient elution, analysts must make compensatory adjustments to one of the other parameters of equation 3. For example, a reduction of flow rate by a factor of two can be balanced with an increase in gradient time by a factor of two. The result of such a change can be observed by comparing Figure 2a with Figure 2c and the corresponding portions of Table II. Although the retention times are roughly doubled, the relative peak spacing (α) is unchanged when flow rate and gradient time are changed simultaneously in this manner. (As in Figure 1, Figure 2c has better resolution than Figure 2a because of a small increase in N when flow is

reduced.) Another way to think about how to keep the separation constant is that the gradient *volume* must be constant. In Figures 2a and 2c, the gradient volume is 40 mL (20 min × 2 mL/min = 40 min × 1 mL/min = 40 mL), whereas Figure 2b has a gradient volume of 20 mL (20 min × 1 mL/min), and the difference in peak spacing is apparent.

If peaks are well separated — for example, resolution is greater than 2 — small changes in only the flow rate (for example, in the range of 10–15%) might make changes in the separation unimportant, even through the selectivity changes. However, when resolution is marginal, as often is the case for stability-indicating or impurity assays, the resolution can be compromised even with small changes in flow rate.

Whenever making changes in gradient conditions, analysts must ensure that they don't cause unintended results. Use equation 3 as a guide. For example, if the column diameter is reduced from 4.6 mm to 2.1 mm, a fivefold reduction in V_m occurs (the column volume is proportional to the cross-sectional area). To compensate for this change, reduce the flow rate or gradient time by a factor of five (or use an appropriate combination of both parameters).

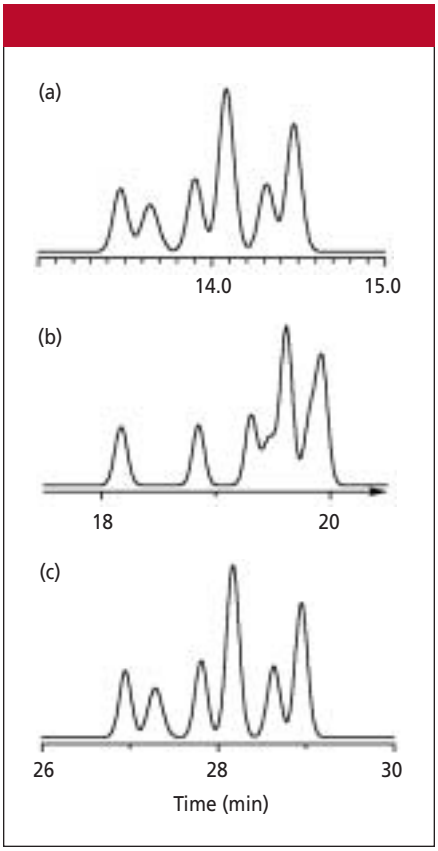

Figure 2: Gradient separation of sample in Table II using (a) a 20-min gradient with a 2-mL/min flow rate, (b) a 20-min gradient with a 1-mL/min flow rate, and (c) a 40-min gradient with a 1-mL/min flow rate.

Conclusion

Mobile-phase flow rate can be changed in isocratic separations without changing relative peak spacing, and it can be a useful tool for making small adjustments in retention to meet system-suitability requirements. When gradient methods are used, however, flow rate can be changed only if another parameter in equation 3 also is changed so that k^* is kept constant.

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